Production of Salt Tolerant Rootstock Lines and Standardization of In Vitro Plant Regeneration Protocol in *Citrus* sp.

A synopsis of the proposed work for
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Submitted by

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INTRODUCTION

Citrus is an evergreen economically important fruit crop cultivated for its juicy fruits rich in vitamin C (Shirgure 2013). The genus *Citrus* (family Rutaceae) includes several species such as pomello, mandarin orange, sweet orange, sour orange, lemon and grapefruit. *Citrus* plant is perennial and medium sized tree. The plant having spiny branches, ovate or elliptic-lanceolate leaves, blossom flowers and hesperidium type fruit (Amington 2011). *Citrus* is native to south-east Asia, Australia and central Africa region (Tanaka 1958; Swingle *et al*., 1967; Mabberley 2004; Jena *et al*., 2009). Major *Citrus* producing countries are Brazil, China, United States, Mexico and India. In India the North-east region is considered to be the centre of origin for citrus (Ghosh 1977 and Govind *et al*., 1999). In India, with regard to cultivated area *Citrus* fruit falls under third largest fruit crop after Banana and Mango. In India, the total land covered by *Citrus* crops is 846 ha with 7464 tones production and 9 tons per ha productivity. Major states contributing largest citrus production in India are Andhra Pradesh, Maharashtra, M. P., Punjab, Gujarat, Karnataka, Assam, Odisha, Rajsthan, Haryana, Uttarakhand, U. P., Arunachal Pradesh (National Horticulture Board, Horticulture Information Service-2010-2011).

Citrus is well known for its nutritional, medicinal and cosmetic properties. *Citrus* fruit is a major source of vitamin C (71 mg per 100g) besides its peel also contains several secondary metabolites as: citric acid, flavonoids, phenolics, pectins, limonoids, ascorbic acids, vitamin E, provitamin A, carotenoids, polysaccharides, lignins, fibre, and essential oil etc. (Iglesias *et al*. 2007). The richness of compounds in citrus imparts it with anti-cancerous properties and is also a strong anti-oxidant. These fruits help in fighting inflammation, allergies and reduce body LDL cholesterol (Dugo and Di Giacomo, 2002 and Iglesias *et al*. 2007).

Rootstocks of some *Citrus* sp. and their wild relatives are used for the production of commercially important *Citrus*. In *Citrus* tree the nucellar embryo is developed through nucellar tissue (an inner most part of ovule in which the embryo sac is pinched off). Since *Citrus* sp. produces polyembryonic seeds through nucellar embryos hence *Citrus* sp. are known to be apomictic (Koltunow *et al*., 1995 *Bouharmont et al*., 1996 and Gloria *et al.* 2000). Development of nucellar embryo is absent in unfertilized ovules while fertilized ovules have ability to develop polyembryonic seeds (Koltunow *et al*., 1995). In several polyembryonic *Citrus* sp. the nucellar embryo develops synchronously to that of zygotic embryo in a seed (Bruck *et al*., 1985; Cameron *et al*., 1968; Kobayashi *et al*., 1979, 1981; Wakana *et al*., 1987, 1988). Nucellar seedlings are being most widely used as source of rootstock for the purpose of propagation.

Citrus production across the world is continuously decreasing. One of the main reason for decrease in yield is the increase in water and soil salinity the world over (Shirgure 2013). Since both vegetative and reproductive growth are highly affected by salinity (Storey and Walker 1999). Studies have shown that saline water has more negative influence on citrus growth than saline soil (Furr *et al*., 1968). Shalhevet and Levy (1990) and Maas (1993) have reported that electric conductivity above 1.4 dS m-1 lead to 13% decrease in yield. Salinity, the main abiotic stress can be defined as, build up of ionized form of different inorganic salts in water and soil. About 7 % of the world's total land area is affected by salinity (Ghassemi *et al*.1995 and Szabolcs 1994). In India, about 8.6 m ha land is affected by salinity (Pathak 2000). In U.P. and surrounding states, almost 2.8 m ha land is salt affected (Abrol *et al.* 1971). These salts causes osmotic stress by increasing water potential of soil; photosynthetic reduction due to decrease in stomatal activity leading to chlorophyll denaturation; and oxidative stress due to ROS

generation causing lipid peroxidation and protein degradation in different fruit crops (McCord 2000).

Natural rootstock named sour orange used across the world is tolerant to Cl^- salt, hybrid rootstock named *Troyer citrange* tolerant to Na salt and citrus jambhiri (rough lemon collection–6 (RLC-6) tolerant to Na and Cl^- salt have been developed. However, rootstocks are not available that are tolerant to different types of salinity at a single place. Therefore, production of new rootstock tolerant to different salinity stresses is needed. Although, breeding programmes play a critical role in crop improvement but till far have failed to develop desirable trait of salt tolerance (Purohit *et al*., 1998). Genetic engineering has become an important tool to improve the crop varieties and produce salt tolerant plants (Borsani *et al*., 2003; Yamaguchi and Blumwald 2005 and Rai *et al*., 2011). However, this technique has certain limitation as non-expression to low level of transferred gene and more importantly poor transformation frequency (Mondal *et al*., 1997). In this context, tissue culture techniques as protoplast fusion and culture have proved to be much more efficient in producing salt tolerant lines (Grosser *et al.* 2000, 2010 and Ollitrault *et al*. 2000).

The present study is aimed at developing suitable salt-tolerant *Citrus* rootstock with following objectives:

1. Production of salt tolerant root stock lines
2. Standardization of *in vitro* regeneration protocol
3. Field establishment of salt tolerant root stock lines and their selection

***** In Uttar Pradesh Agra and Mathura is included under most saline city, present status of EC of irrigation water in Agra district is 2.5 – 3.0 ds/m which is much greater as per US guide (0.7 ds/m) and also increasing day by day (Gupta *et al*. 2013). Thus the present research wish to complete above objectives for the following deliverable expected:

- Salt tolerant rootstock will be developed that can grow better with higher yield at high salt concentration, hence citrus production can be increased in highly saline regions.
- Through cell line selection salt tolerant *in vitro* rootstock of desired citrus could be directly produced.
- Through standardization of regeneration protocol, *in vitro* seedlings of salt tolerant *Citrus* can be produced in large number.
- This idea will also help to improve other salt sensitive crop plants.
- *In vitro* establishment of cultures will also make ease of plant material availability for further laboratory research to improve other aspects in *Citrus.*

LITERATURE REVIEW

Since 1980s somatic hybridization via protoplast fusion has become a most powerful tool for crop improvement by producing new allotetraploid hybrid rootstock with complementary characteristics (Grosser 1997, 1998 and Louzada 1992). First case of somatic hybridization via protoplast fusion of intergeneric and sexually compatible *Citrus* cv. was reported by Ohgawa *et al*. (1985) who produced allotetraploid somatic hybrid from *C. sinensis* (sweet orange) and *Poncirus trifoliata* and nowdays somatic hybridization for cv. improvement has been started in several countries including US, France, Japan, Irasel, India and China etc. at large scale. Till date more than 150 *Citrus* somatic hybrids have been reported (Ohgawa *et al*., 1994 and Grosser 1995). Since this technique facilitate the production of allotetraploid and autotetraploid hybrid hence also promoting conventional breeding programmes and genetic transformation for ploidy manipulation by combining entire nuclear genome of complementary parents. Several strategies have been employed for rootstock improvement: Production of somatic hybrid from complementary rootstock parents have potential for disease resistant, tree size control, biotic and abiotic stress tolerance and horticultural performance; produce somatic hybrid with wild relatives that are sexually incompatible or difficult to hybridize with citrus relatives for germplasm enhancement and production of somatic cybrid have potential for both scion and rootstock improvement (Grosser *et al*., 2000 and 2011). Although complete analysis of new rootstock candidates is time consuming, expensive process requires much labour and co-operation of horticulturists, pathologists, nematologists and commercial growers and also requires propagation of hundreds of each hybrid plant for complete evaluation yet somatic hybrid rootstock have been produced (Grosser *et al*., 2000).

Callus established directly from explant and newly established suspension from immature callus cultures is consist of highly starchy cells and this starch interferes with the yield and viability of protoplast. It also resists the fusion of protoplast when young callus or suspension culture used for protoplast fusion and mostly diploid or unfused plants obtained. Hence in earlier studies habituated callus (more than three years) was preferred because habituated callus and suspension culture loosed its totipotency and promotes cell fusion thus percentage of tetraploid increased (Grosser *et al*., 2011).

To produce intergeneric somatic hybrid Grosser *et al*. (1987, 1992, 1996) used suspension culture, Grosser *et al*. (1990, 1992 and 1996); Gmitter *et al*. (1992) and Dambier *et al*. (2011) used juvenile stem, nucellar tissue, ovule, style or seed derived callus of one parent maintained on MT basal + 500 mgL^{-1} malt extract and 50 mgL^{-1} sucrose and leaves of other parent to isolate protoplast. Grosser *et al*. (2011) have initiated callus from ovule culture on DOG medium (EME+5mgL^{-1} kinetin) was habituated on EME or H+H medium without growth regulator. SSR genetic marker was used for identification of zygotic or nucellar origin of callus. Suspension culture was initiated and maintained from habituated callus on liquid H+H medium. While in case of *Severinia disticha* epicotyls derived callus was used in place of leaves for protoplast isolation and maintained on MT basal medium with different hormone concentration Grosser *et al*. (1988). Cells used for protoplast isolation should be in log phase of growth. In earlier publications BH_3 medium and enzyme solution was used for protoplast isolation and sucrose mannitol gradient protocol was used for protoplast purification using 45μm stainless steel sieve and several centrifugation steps at 100g Grosser et al. (2011). PEG mediated fusion of protoplast was done on BH_3 medium (Menczel *et al*., 1981 as given in Grosser *et al*., 1987, 1988, 1990, 1996 and 2011) and after fusion protoplast culture on BH_3 or EMEP medium or both BH_3

and EMEP (1:1) Grosser *et al*. (1996, 2007). For embryogenesis and embryo germination MT medium with gradually reduced sucrose was used by Grosser *et al*. (1987, 1988, and 1990); solid EME maltose medium used by Grosser *et al*. (2007 and 2011) for embryo development and medium used for germination and rooting was B and RAMAN respectively B+ or DBA_3 medium was used by Grosser *et al*. (1996) for embryo germination.

Although somatic embryogenesis from callus culture reported on EME basal + 25 gL^{-1} maltose medium (Perez *et al*., 1998) but embryo enlargement found to be maximum on medium without maltose and reduced sucrose stating that low sucrose concentration influences embryo development while suppresses calli growth. GA_3 1 mg L^{-1} have positive effect on embryo germination (Mendes-Da-Glória *et al*., 2000). Earlier studies shows that only hybrid cell are able to grow at high sucrose concentration (Ohgawa 1985; Motomura 1995 and Mendes-Da-Glória *et al*., 1999 and 2000).

According to Grosser *et al*. (1987) *Poncirus, Severinia* and *Citropsis* are wild relative of *Citrus* having most potential germplasm tolerant to both biotic and abiotic stresses. ***Poncirus*** is known to be a potential germplasm source of cold hardiness, resistant to Citrus Tristeza Virus (CTV), Foot rot (*Phytophthora parasitica*) and Citrus Nematode (*Tylenchulus semipenetrans*); ***Severinia*** is also a germplasm source of cold hardiness, salt and Boron tolerance and ***Citropsis*** is resistant to diseases and pests.

Grosser *et al*. (1987) obtained allotetraploid somatic hybrid (2n = 4x = 36) by fusing embryogenic suspension culture derived protoplasts of *Citrus sinensis* cv. Hamlin (Sweet orange) with leaf derived protoplasts of *Poncirus trifoliate* cv. Flying Dragon (Trifoliate orange). Grosser *et al*. (1988) developed another allotetraploid from sexually incompatible trees by chemical fusion of embryogenic suspension culture derived protoplast of *C. sinensis* with

protoplast of *Severinia disticha* isolated from in vitro epicotyl derived callus. In 1990, Grosser *et al*. obtained somatic hybrid by combining the *Citropsis gilletiana* genome (a graft compatible but sexually incompatible germplasm to *Citrus*) resistant to *Phytophthora citropsis* causing foot rot and burrowing Nematode (*Radopholus citropsis*) with sweet orange and for this embryogenic suspension derived protoplast is fused with protoplast from different source (young leaves and young stem derived callus) of *Citropsis gilletiana* and maximum somatic hybrid embryo were recovered with leaf derived protoplast rather than callus hence results are entirely different from Grosser *et al*. (1988).

Later on these six new somatic hybrid have been produced viz. *Citrus sinermis* (L.) Osbeck cv. Hamlin + *Severinia buxifolia* (Poir.) Tenore (Chinese box-orange); C. *reticulate* Blanco cv. Cleopatra + *Poncirus trifoliata* (L.) Raf. cv. Flying Dragon; C. *reticulate* cv. Cleopatra + Swingle citrumelo (C. *paradisi* Macf. × *P. trifoliata); C. sinensis* cv. Hamlin + C. *jambhiri* cv. Rough lemon; C. *sinensis* cv. Valencia + C. *jambhiri* cv. Rough lemon; and C. *paradise* cv. Thompson + 'Murcott' tangor (purported hybrid of C. *reticulate* x *C. sinensis*). Diploid plants obtained from non-fused embryogenic culture-derived protoplasts of 'Cleopatra' mandarin and sweet orange and leaf derived protoplast of Murcott and Rough lemon. Fusion product of *Citrus sinermis* cv. Hamlin + *Severinia buxifolia* not tetraploid when chromosome number and ploidy level of regenerated plant was studied and it was found that somatic hybrid of *Citrus sinermis* CV. Hamlin + *Severinia buxifolia* have (2n=27) which can be triploid or aneuploid Grosser *et al*. (1992). Gmitter *et al*. (1992) have also produced following eight new somatic hybrid citrus rootstocks - *Citrus reticulata* Blanco (Cleopatra mandarin) + *C. aurantium* L. (sour orange), *C. reticulata* (Cleopatra mandarin) + *C. jambhiri* Lush (rough lemon), *C. reticulata* (Cleopatra mandarin) + *C. volkameriana* Ten. & Pasq. (Volkamer lemon), *C.*

reticulata (Cleopatra mandarin) + *C. limonia* Osb. (Rangpur), *C. sinensis* (L.) Osb. (Hamlin sweet orange) + *C. limonia* (Rangpur), *C. aurantium* (sour orange) + *C. volkameriana* (Volkamer lemon) zygotic seedling, *C. auruntium* hybrid (Smooth Flat Seville) + *C. jambhiri* (rough lemon), and *C. sinensis* (Valencia sweet orange) + Carrizo citrange [*C. paradisi* Macf. × *Poncirus trifoliata* (L.) Raf.] using protocol of Grosser *et al*. (1990). In 2000, Grosser *et al*. produced 19 new somatic hybrid rootstock.

Somatic hybrid of sour orange + sweet lime and sour orange + *Carrizo citrange* (Grosser *et al*. 1996) was found resistant to Citrus Tristeza Virus (CTV), Citrus Blight and Phytophthora induced disease. In 2000, Grosser *et al*. produced 19 somatic hybrid rootstock and *Citrus aurantium* (sour orange) is nowadays most common rootstock used for saline condition. Rootstock Cleopatra + *Swingle citrumelo* was produced for reduced tree size with higher yield, upon field trial trees budded on this rootstock were found small sized and comparatively higher yield. Trees budded on somatic hybrid of sour orange + Palestine (sweet lime) have higher yield than tree on root of sour orange (common rootstock in Florida) alone. New rootstocks via protoplast fusion of Cleopatra + Argentine trifoliate orange; sour orange + *Carrizo citange* and sour orange + Palestine sweet lime have ability to control tree size, disease resistance and good quality fruits. Hybrid plants (*Citrus deliciosa* + *Poncirus trifoliata*) are resistant to CTV, Phytophthora and well adapted for calcareous soil and under high-saline condition. Till date no somatic hybrid have been produced for resistant to Diaprepes root weevil larvae, various nematode and salinity. Mendes-Da-Glória *et al*. (2000) have also developed somatic hybrid of sweet orange Caipira (*C. sinensis* L. Osbeck) and Rangpur lime (*C. limonia* L. Osbeck) tolerant to drought and blight. New Rootstock [White grapefruit + trifoliate orange; Changsha + trifoliate orange; sour orange + Carrizo and Changsha + Benton] were developed with better adaptability

to various soils (highly alkaline, high pH and calcareous soil) than the earlier rootstocks (sour orange + Flying Dragon and sweet orange + Flying Dragon) developed before Grosser *et al*. (2005 and 2011). To produce new rootstock Grosser *et al*. (2007) have developed sting nematode tolerant Pumello plant to be used as parent and then its leaf derived protoplast was allowed to fuse with suspension culture derived protoplast of local mandarins and tetraploid hybrid obtained was resistant to nematode and have good nursery characteristics. Due to zygotic embryony Pumello is resistant to salinity, Phytophthora, Diapreps and adaptation to various soil Grosser (2003 and 2004) and Ananthakrishnan (2006).

Earlier studies confirmed that hybrid obtained via protoplast fusion was tetraploid showing intermediate type phenotype (Ohgawa *et al*., 1985; Grosser *et al*., 1985, 1987, 1988, 1990, 1992, 2000, 2011; Gmitter *et al*., 1992; Motomura 1995 and Mendes-Da-Glória *et al*., 1999 and 2000). To confirm hybrid nature leaf morphology of regenerant was compared with the parent plants, chromosome number and ploidy level of regenerant was confirmed by flocytometer and electrophoretic analysis for isozymes activity Grosser *et al*., 1987 (MDH), 1988 (MDH and Phosphoglucomutase), 1992, 1996 and Vallejos (1983) (Peroxidase, Phosphoglucomutase and Phosphoglucoisomerase) 2011; Gmitter *et al*., 1992; Ling and Iwamassa 1994). Chromosome number was determined by Hematoxylin – staining protocol (grosser *et al*., 1990 and 1996). Allotetraploid somatic hybrid between citrus and related genera have been produced and confirmed by PCR analysis for RAPD markers. Strong evident for hybrid nature of tetraploid was PCR-RAPD banding pattern showing expression of gene or DNA sequences from both parents (Grosser *et al*., 1996 and Mourão Filho *et al*., 1996) and rDNA restriction endonuclease digestion pattern (Ohgawa *et al*., 1985 and Miranda *et al*., 1997).

Autotetraploid and allotetraploid are also determined by using RAPD and EST-SSR Grosser *et al*. (2011).

These somatic hybrid were released for commercial trial where were found to be resistant to foot rot (Grosser *et al*., 1990). Somatic hybrids of Hamlin + *S. buxifolia;* Cleopatra + Trifoliate orange and Cleopatra + *Swingle citrumelo* released for field trial and were found to have potential for citrus scion and rootstock improvement for commercial application. Somatic hybrid of Thompson + Murcott widely used as tetraploid breeding parent for scion improvement and for phenotypic stability of mature tree, its leaf protoplast derived diploid plants were grafted to this rootstock Grosser *et al*. (1992). Gmitter *et al*. (1992) developed somatic hybrid rootstocks have been propagated and entered into field trials for determining their characteristics and nature of genetic control of important traits and selection of parents for further somatic hybridization.

For production of salt tolerant rootstock *in vitro* selection procedure with suitable selection agent is also recommended to use. For salt tolerant rootstock NaCl is used as selecting agent (Purohit *et al*. 1998). According to Purohit *et al.* (1998) and Mohamed *et al.* (2000) only tolerant genotype is able to grow on the selection medium.

In 2008, Patil *et al*. have successfully developed mango rootstock through grafting. Omba *et al*. (2010) describe the suitable size of stock and scion to produce successful rootstock via grafting experiment. In this context, it is assumed that micro grafting technique describe above will be suitable to produce salt tolerant rootstock in short time period.

PROPOSED METHODOLOGY

Plant Material

Plant material for the present study will be selected from known commercial *Citrus* sp. and their wild related genera:

Commercial Citrus sp.

1. ***Citrus sinensis* L. (Sour orange),** most common rootstock used throughout the world due to its adaptability to variety of soils and has Cl^- exclusion property. Besides, the species is cold hardy, resistant to *Phytophthora* and citrus blight. However, it is highly susceptible to Citrus Tristeza Virus (CTV) (Grosser *et al*. 2000 and 2011).
2. ***Citrus sinensis* L. (Osb.) (Hamlin sweet orange),** resistant to citrus blight but highly susceptible to *Phytophthora* induced diseases and salinity hence not used as rootstock (Grosser *et al*. 1992).
3. ***Citrus jambhiri* (Rough lemon RLC-6),** this rootstock has Na and Cl^- exclusion property and resistant to CTV and drought. However, this line is extremely susceptible to citrus blight (Grosser *et al*. 1990 and 2000)

Related genera

1. ***Poncirustrifoliatae* (Flying dragon Trifoliate orange),** wild relative of citrus highly susceptible to salt but resistant to CTV and Phytophthora (Grosser et al. 1987).
2. ***Troyer citrange* (Hybrid of *Poncirustrifoliatae* x sweet orange)** have Na exclusion property but highly susceptible to salt. These lines are resistant to CTV, Phytophthora, and citrus blight.

- All these lines will be available for IARI, New Delhi.

The proposed methodology for the work given below:

- **Protocol to establish *in vitro Citrus* cultures:**
  - Nucellar embryos of sour orange, sweet orange, trifoliate orange, troyer citrange and rough lemon will be used as source of rootstock to establish *in vitro* callus cultures.
  - Callus culture will be maintained by subculturing at an interval of 30 days on appropriate callus induction medium.
- **Protocol to produce salt tolerant rootstock**
  - In present study salt tolerant rootstock will be produce by following three techniques:

1. **Through salt tolerant rootstock lines:**
   - Callus cultures of sour orange, sweet orange, trifoliate orange and troyer citrange will be subculture on MS medium with a gradient of NaCl.
   - Salt tolerant cell lines of sour orange, sweet orange, trifoliate orange and troyer citrange will be selected and transfered to regeneration media maintaining the salt concentration.
   - Regenerated plants will be rooted and hardened under high salt concentration in the field.
2. **Through protoplast fusion:**
   - Protoplast from salt sensitive callus of sour orange, sweet orange and trifoliate orange and salt tolerant callus of rough lemon and will be isolated and fused

through various methods. Thereafter, heterokaryon will be selected for recovery of salt tolerant plant followed by field acclimatization.

3. **Through micrografting:**
   - In this technique the scion of the susceptible parent will be graft on salt tolerant rootstock under *in vitro* conditions.
   - If grafting succeeds, from the graft region callus or tissue will be collected to develop *in vitro* plants. The regenerated plants will be screened for salt tolerance.

Protocol for selection of salt tolerant rootstock lines of *Citrus*

For selection of salt tolerant lines following parameters will be used:-

1. **Plant biomass**

   Salt tolerant and salt sensitive recovered plant's biomass will be evaluated by measuring fresh weight, dry weight, thickness of leaf, length and thickness of root and shoot and compared.

2. **Physiological parameters**

   For physiological parameters leaves of recovered plants (salt tolerant and sensitive) will be selected to calculate number of stomata, their size, number of epidermal cells, photosynthetic rate and water use efficiency.

3. **Level of ions**

   Level of Na^+ , Cl^- and other ions will be measure in different parts of plants (leaf and root) by the method describe by Wolf (1990) and followed by Balal *et al* (2012) .

4. Activity of Anti-oxidant enzymes

Activity of superoxide dismutase enzyme will be determine by using protocol of Giannopolitis and Ries (1977) and activity of enzyme catalase and peroxidase will be measure by using protocol of Chance and Maehly (1955).

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